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Correlation Between ELISA, Hemagglutination Inhibition, and Neutralization Tests After Vaccination Against Tick-Borne Encephalitis

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The significance of IgG antibody levels determined by a binding assay (ELISA) was investigated as a surrogate marker for the presence of neutralizing and hemagglutination inhibiting antibodies in sera from individuals vaccinated against tick-borne encephalitis (TBE). To assess the extent of interference by flavivirus cross-reactive antibodies, sera from persons with a proven or suspected history of other flavivirus infections and/or vaccinations were also examined. An excellent and highly significant correlation was found between ELISA IgG units and the antibody titers obtained by the hemagglutination inhibition (HI) as well as by the neutralization test (NT), provided that there was no other exposure to flavivirus antigens except TBE vaccination. Yellow fever vaccination and/or dengue virus infections induced significant levels of antibodies reactive in the TBE ELISA and HI test, which did not exhibit, however, neutralizing activity against TBE virus. The phenomenon and problem of "original antigenic sin" was demonstrated in a TBE vaccinee with a history of previous flavivirus infections. TBE vaccination first induced a booster reaction resulting in a rise in the level of cross-reactive antibodies only, whereas TBE virus-neutralizing antibodies became detectable only after the third vaccination. It is concluded that the level of IgG antibodies determined by ELISA is a good marker for predicting the presence of neutralizing antibodies after TBE vaccination, but only in the absence of flavivirus cross-reactive antibodies. Otherwise, a neutralization assay is necessary for assessing immunity.

KEY WORDS: antibodies after TBE vaccination, original antigenic sin, interference by cross-reactive antibodies

INTRODUCTION

Enzyme immunoassays have become the method of choice for the rapid serodiagnosis of virus infections by the detection of specific IgM antibodies. They also have been applied in a quantitative assay format to monitor immune responses to vaccines such as hepatitis B and hepatitis A [Ostrow et al., 1991; Van Damme et al., 1994], and the level of specific IgG determined by ELISA has been used as a marker for immunity and to estimate necessary booster intervals. The value of quantitative IgG antibody determination by ELISA after vaccination against tick-borne encephalitis (TBE) was investigated by examining the correlation between ELISA antibodies and neutralizing antibodies in post-vaccination sera. Compared to hepatitis B or A virus, for which there is only one serotype, the problem is significantly more complex with TBE virus, because it is related antigenically to several other viruses that infect humans, and the interference in ELISA of cross-reactive but nonneutralizing antibodies should be considered.

TBE virus is a member of the genus flavivirus within the family *Flaviviridae*, which comprises 70 distinct but antigenically related viruses. Flaviviruses are small spherical, lipid-enveloped viruses that contain three structural proteins: C (capsid), M (membrane), and E (envelope) [for review see Chambers et al., 1990]. Protein E covers the virion surface, is the viral hemagglutinin, and is the primary target for neutralizing antibodies. All flavivirus E proteins share common antigenic sites as revealed originally by cross-reactivity studies using hemagglutination inhibition (HI) tests [Casals, 1957]. By the more discriminating cross-neutralization assay, however, flaviviruses can be subdivided into distinct serocomplexes [DeMadrid and Porterfield, 1974; Calisher, 1989]. This subdivision is also

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TABLE I. Comparison Between ELISA, Hemagglutination Inhibition, and Neutralization Tests

ELISA (VIE units)	n	Percentage positive HI	NT_{100}	NT_{50}
– (<63)	20	0	0	0
+/– (63–126)	37	32.4	32.4	94.6
+ (>126)	157	94.3	80.9	93.6

reflected in evolutionary trees based on sequence comparisons of the E protein [reviewed by Heinze et al., 1990].

The most important human pathogenic flaviviruses are Yellow fever (YF), Dengue (Den), Japanese encephalitis (JE), and TBE virus [for review see Monath, 1990]. Vaccines are used widely for prophylaxis of YF (live attenuated), JE, and TBE (both formalin-inactivated whole virus) [reviewed by Stephenson, 1988]. Vaccination against TBE has been shown to be extremely effective for preventing disease. Seroconversion rates of 97–100% (depending on the age of the vaccinees) after three or more vaccinations were reported, and the protection rate in the field was estimated to be 98% [Kunz, 1992].

TBE virus-specific antibodies can be quantitated by ELISA using purified virus as an antigen [Hofmann et al., 1983]. Since the virion surface is formed by a network of protein E dimers [Allison et al., 1995; Rey et al., 1995], this ELISA is expected to indicate the presence of type-specific and potentially neutralizing antibodies, but also that of flavivirus cross- reactive, nonneutralizing antibodies.

In the present study, TBE postvaccination sera were examined and the correlation between ELISA, HI, and neutralizing antibodies was evaluated. The problem of flavivirus cross-reactive antibodies was addressed specifically by testing sera from YF vaccinees and persons with a proven or suspected history of dengue virus infections. In the absence of cross-reactive antibodies, there was an excellent and highly significant correlation between ELISA IgG units and NT (neutralization test) as well as HI titers. YF vaccination and/or dengue infections induced significant levels of antibodies reactive in the TBE ELISA, which, however, did not neutralize TBE virus. It is concluded that the level of postvaccination IgG antibodies determined by ELISA is a good marker for predicting the presence of neutralizing antibodies against TBE virus, but only in persons without a history of other flavivirus vaccinations or infections.

MATERIAL AND METHODS

Clinical Specimens

The 269 sera examined in this study included 194 TBE postvaccination sera, 20 negative sera from unvaccinated individuals, and 51 sera were from U.S. and other NATO troops with a proven or suspected history of other flavivirus infections or YF vaccination [Clement et al., 1992, 1994]. Four serum specimens were obtained from one individual who had been vaccinated against YF in 1975 and 1980 and who had suffered from a Den-1 virus infection in 1989 prior to the TBE vaccination in 1993. All of the sera were stored at – 20°C.

Neutralization Assay

For the NT, a 20% (w/v) suckling mouse brain suspension of TBE virus strain Neudoerfl [Mandl et al., 1988] was used, and infectivity titrations of this suspension were carried out by plaque assay using PS (procine kidney) cells. The test was performed in 96 flat-well microtiterplates. Serum samples were titrated in duplicate in twofold dilution steps (50 μl/well), starting at a dilution of 1:10 in MEM Earle's medium containing 1% fetal calf serum, 1% glutamine, and 1% neomycin. Equal volumes of TBE virus and serum dilutions in MEM-Earle's were mixed to give a final virus concentration of 1,000 PFU/ml. These mixtures were incubated for 1 hour at 37°C. A BHK-21 cell suspension ($1x10^5$ cells in a volume of 100 μl) was then added and the cells were incubated at 37°C for 4 days. On day 4 p.i. (postinfection), 50 μl aliquots from the supernatants were tested at a 1:3 dilution for the presence or absence of viral antigen in a four-layer ELISA system (see below). The NT titers (NT_{100}) were expressed as the reciprocal of the serum dilution that was able to suppress virus infection to such an extent that no viral antigen could be detected in the supernatant by ELISA (OD at 450 nm <0.1). The test was repeated twice and the results averaged. The serum dilution required for a 50% reduction of the antigen ELISA value was also determined (NT_{50} titer). NT_{100} titers ≥10 and NT_{50} titers >10 were considered positive. In each test, titrations of virus controls in the absence of antibodies and three positive and one negative serum controls were included.

ELISA Procedures

TBE virus antigen was quantified by four-layer ELISA as described by Heinz et al. [1986], using guinea pig anti-TBE IgG as capture antibody and rabbit anti-TBE IgG in combination with peroxidase-labeled horse anti-rabbit IgG as the detection system.

TBE virus-specific IgG antibodies in serum samples were detected by a three-layer ELISA on 96-well microtiterplates as described previously [Hofmann et al., 1983]. As an antigen, purified TBE virus strain Neudoerfl [Heinze and Kunz, 1981] (1 μg/ml in carbonate buffer pH 9.6) was coated onto the solid phase and the sera were tested at a dilution of 1:100. Peroxidase-labeled goat antihuman IgG (Nordic) was used as detecting antibody and absorbance was measured at 450 nm. Sera were quantitated in Vienna (VIE) units using a standard human anti-TBE antiserum [Hofmann et al., 1983]. ELISA values ≥127 VIE units were considered positive and 63–126 VIE units borderline.

Hemagglutination Inhibition (HI) Tests

HI tests were carried out at pH 6.4 with goose erythrocytes essentially according to Clarke and Casals [1958] using acetone-extracted antigen from mouse brain infected with TBE virus (strain Neudoerfl), Den-2 virus (strain TR 1751), YF virus (strain Asibi C-738), or Murray Valley encephalitis (MVE) virus at a concentration of four hemagglutinating units. Serum titrations were carried out in twofold dilution steps starting at a dilution of 1:10. Titers ≥ 10 were considered positive.

Statistical Analysis

The correlation between ELISA units and NT or HI titers was assessed based on a random sample of positive TBE postvaccination sera. Weakly positive postvaccination sera were especially selected for the sensitivity analysis. Pearson correlation coefficients were computed after logarithmic transformation of ELISA units and titers. Normality of transformed values was tested by Kolmogorov-Smirnov tests.

Logistic regression analysis of NT- and HI-positive results vs. ELISA units was performed on the total sample. Goodness of fit was tested based on likelihood ratio statistics.

RESULTS

Correlation Between ELISA, Hemagglutination Inhibition, and Neutralization Tests

In order to investigate the significance of ELISA results for evaluating the immune response to TBE vaccination, the correlation was examined between the level of virus-binding antibodies as measured by ELISA with the levels determined in functional assays, i.e., HI and NT. For this purpose, 157 randomly selected ELISA-positive postvaccination sera (i.e., containing >126 VIE units), a specially selected group of 37 sera that were only borderline positive (63–126 VIE units), and 20 negative sera were tested. Two different criteria were employed for determining endpoints in the neutralization assay (NT_{100} and NT_{50} titers) as described in Materials and Methods, and percentages of positive results obtained in the functional assays are given in Table I. As shown in Table I, the number of samples scoring positive was significantly increased by applying the NT_{50} criterion, which became especially apparent in the low and borderline IgG range. The correlation between ELISA units and HI as well as NT titers are shown in Figure 1A–C. There was a highly significant correlation between ELISA units with HI, NT_{100}, and NT_{50} titers ($r = 0.84$ ($P < 0.00001$), $r = 0.81$ ($P < 0.00001$), and $r = 0.65$ ($P < 0.00001$), respectively).

Logistic regression analysis was also performed to determine the relation between ELISA units and the probability of a positive result in HI and NT_{50} (goodness of fit: Chi-square = 65.3; df = 1 for NT_{50}, and chi-square = 67.5; df = 1 for HI; both $P < 1 \times 10^{-6}$). As can be seen in Figure 2, the 50% probability of a positive NT_{50} assay corresponds to 64.3 ELISA units, and of a positive HI to 119.2 units.

Problem of Flavivirus Cross-Reactive Antibodies

To evaluate the extent of interference by flavivirus cross-reactive antibodies in the different test systems, serum samples were tested from 51 soldiers from U.S. and other NATO troops stationed in Germany, most of whom had been vaccinated against YF and/or had lived in an area endemic for dengue fever, but did not have a history of TBE vaccination. Thirty-eight out of 51 were positive by the ELISA with a mean IgG value of 415 units. As shown in Table II, 20 of these ELISA-positive sera were also positive by the TBE HI test, but none of the 51 sera exhibited neutralizing activity against TBE virus, even when the less stringent NT_{50} criterion was applied.

Formation of Antibodies in a TBE Vaccinee With a History of Flavivirus Infections

The following case report exemplifies the problem of monitoring successful vaccination against TBE by ELISA in an individual with preexisting flavivirus cross-reactive antibodies due to YF vaccination and infection with Den-1 virus. A 58-year-old female with a history of two YF vaccinations in 1975 and 1980 and a serologically confirmed Den-1 virus infection in 1989 received three doses of TBE vaccine in 1993 at intervals of 9 and 41 days. Serum samples were taken on day 0 (the day of the first vaccination), at the time of the second and third vaccinations, and 24 days after the third vaccination. The sera were analyzed in the TBE ELISA and NT, and in HI using TBE, Den-2, YF, and MVE antigens. As shown in Table III, the prevaccination serum was positive in the TBE ELISA, contained relatively high HI antibody levels against Den-2, YF, and MVE, but was negative in the TBE NT. This pattern did not change significantly after the first vaccination, but after the second vaccination considerable titer increases were observed by the TBE ELISA and HI. Simultaneously, the HI antibodies, especially against YF, but also those against Den-2 and MVE virus were strongly increased, whereas the TBE NT was still negative. Neutralizing antibodies against TBE virus became detectable only after the third vaccination.

DISCUSSION

This study demonstrated that the quantitative determination of specific IgG in TBE postvaccination sera by ELISA exhibits an excellent correlation between HI- and NT titers. This may be due to the fact that the ELISA antigen employed consisted of highly purified whole virions covered with an oligomeric network of protein E [Allison et al., 1995; Rey et al., 1995], which represents the primary target for HI and neutralizing

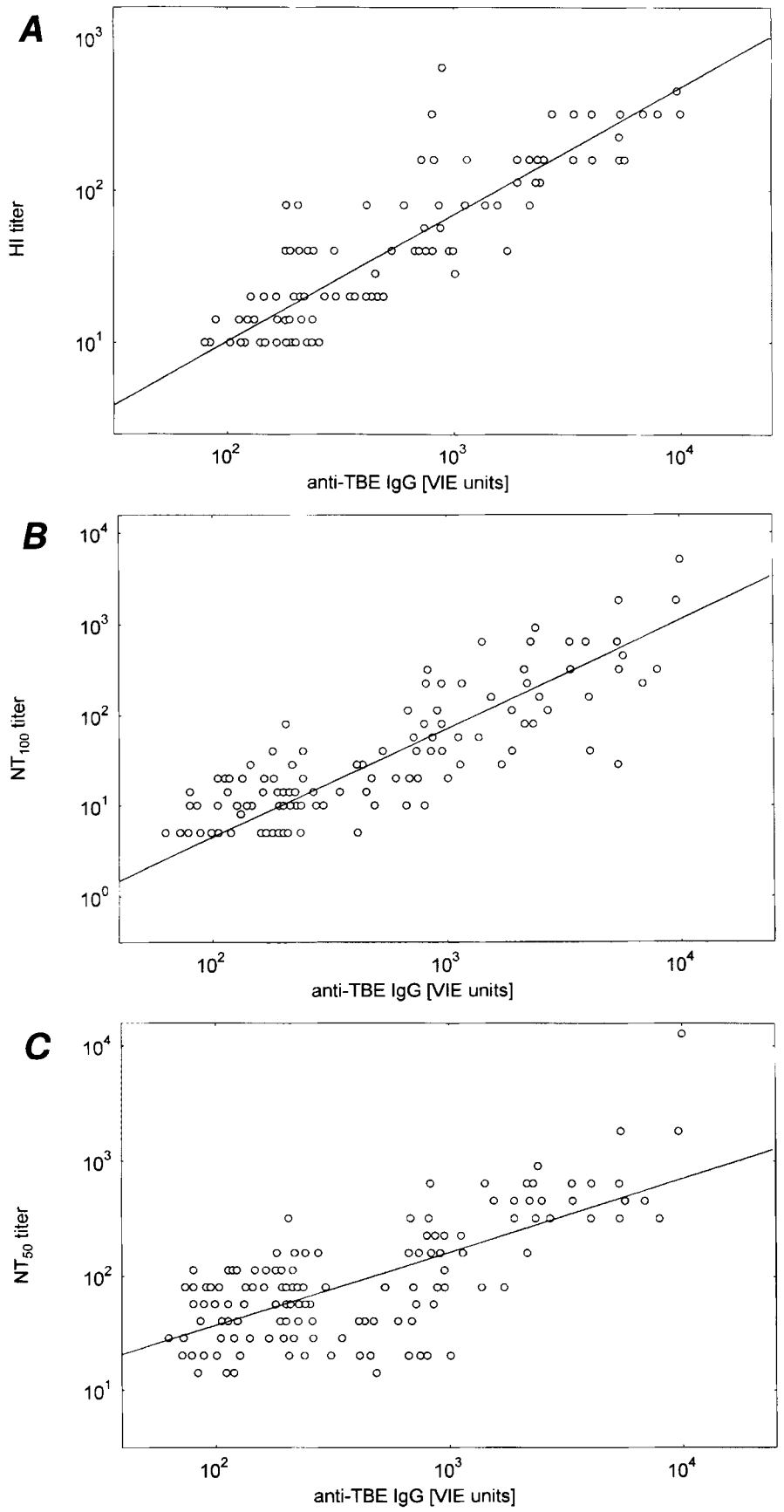

Fig. 1. Correlation between (**A**) anti-TBE IgG units (ELISA) and HI titers; (**B**) IgG units and NT_{100} titers; and (**C**) IgG units and NT_{50} titers.

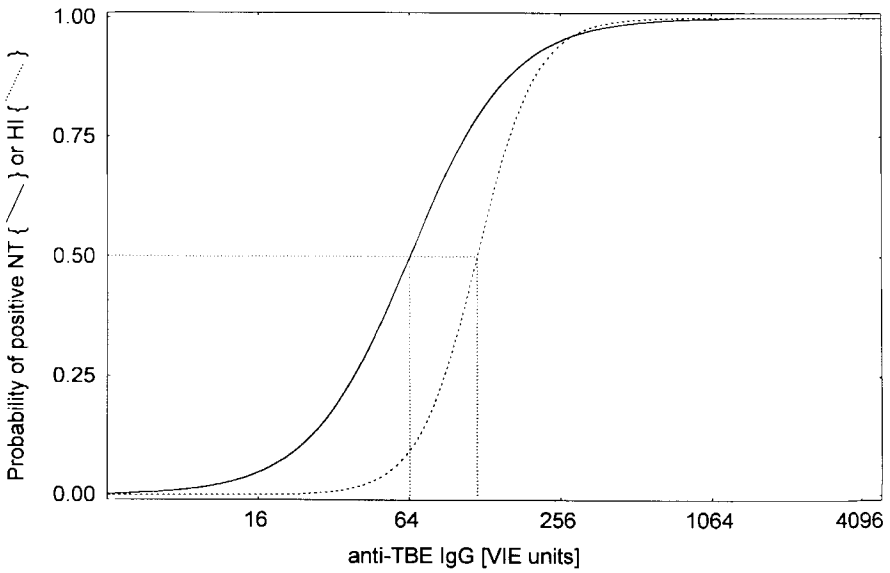

Fig. 2. Probability of positive neutralization- and hemagglutination inhibition test results in relation to IgG units.

TABLE II. Analysis of Sera From NATO Soldiers Without History of TBE Vaccination in TBE ELISA, Hemagglutination Inhibition, and Neutralization Test

	Number of sera positive (%)		
n	ELISA	HI	NT
51	38[a] (74.5)	20 (39.2)	0 (0)

[a]Geometric mean titer (gmt): 415 VIE units.

antibodies. The presence of specific antibodies in this ELISA is therefore highly predictive for the presence of neutralizing antibodies, and this correlation can probably be used in the future to establish guidelines for recommendations of booster vaccinations similar to those used for hepatitis A and B vaccinations.

Two different criteria were employed for determining endpoints in the neutralization assay. NT_{100} titers correspond to the serum dilution that prevented virus replication completely, whereas NT_{50} titers to the dilution that reduced the virus yield by 50%. NT_{100} and HI exhibited similar sensitivities, whereas the NT_{50} also allowed the detection of neutralizing antibodies in sera with low ELISA titers. Specifically, in a group of 37 sera classified as "borderline" in ELISA, 94.6% proved to be positive in the NT_{50}. This figure is similar to the percentage of NT-positives in unambiguously ELISA-positive postvaccination sera and approaches the figure of the protection rate in the field, which was estimated to be 98–99% [Kunz, 1992]. In interpreting these data, it must be kept in mind, however, that most of the sera represented a random collection of samples obtained after an unknown number of vaccinations and after an undefined time point after the last vaccination. It is also emphasized that the weakly ELISA-positive samples were overrepresented, since they were selected specifically by this criterion.

Cross-reactive antibodies induced by other flavivirus infections and/or vaccinations represent a major obstacle for using the level of ELISA antibodies as a surrogate marker for neutralizing antibodies. This is an increasing problem in Europe, because the popularity of travel to tropical and subtropical countries where other flaviviruses are endemic has resulted in a growing number of individuals being vaccinated against YF or JE and in the increased chance of acquiring other flavivirus infections such as dengue [Monath, 1994].

As exemplified by the case report described in this report, pre-existing immunity to one or several flaviviruses in combination with TBE vaccination results in an "original antigenic sin" phenomenon, which was first observed for immune responses to sequential infections or immunizations with different types of influenza virus [Francis, 1953; Davenport and Hennessy, 1956], but which also has been described for sequential flavivirus infections [Halstead et al., 1983; Inouye et al., 1984]. A rise in the level of TBE ELISA-reactive antibodies may thus not necessarily mean that type-specific and neutralizing antibodies were formed, but can be due to the booster of cross-reactive antibodies only. Due to its simplicity and ease of automation, the ELISA will undoubtedly remain the first choice for monitoring immune responses to TBE vaccination. Its combination with the NT, however, should be considered in specific cases where the presence of cross-reactive antibodies due to other flavivirus infections or vaccinations cannot be excluded. The NT is also required to confirm positive ELISA results obtained in serological surveys conducted in areas where TBE virus has not been previously shown to be endemic.

TABLE III. Formation of Antibodies in a TBE Vaccinee With a History of Flavivirus Infections

Blood sample no.	Day	TBE vaccin.	TBE IgG[a]	HI titers against				TBE NT titers	
				TBE	Den-2	YF	MVE	NT_{100}	NT_{50}
1	0	1st	454	10	80	320	80	0	0
2	9	2nd	490	10	80	320	80	0	0
3	50	3rd	>1,000	40	320	>1,280	160	0	0
4	74	none	>1,000	80	320	>1,280	320	40	80

[a]In VIE units.

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REFERENCES

Allison SL, Schalich J, Stiasny K, Mandl CW, Kunz C, Heinz FX (1995): Oligomeric rearrangement of tick-borne encephalitis virus envelope proteins induced by an acidic pH. Journal of Virology 69:695–700.

Calisher CH, Karabatsos N, Dalrymple JM, Shope RE, Porterfield JS, Westaway EG, Brandt WE (1989): Antigenic relationships between flaviviruses as determined by cross-neutralization tests with polyclonal antisera. Journal of General Virology 70:37–43.

Casals J (1957): The arthropod-borne group of animal viruses. Transactions of the New York Academy of Sciences 19:219–235.

Chambers TJ, Hahn CS, Galler R, Rice CM (1990): Flavivirus genome organization, expression, and replication. Annual Reviews in Microbiology 44:649–688.

Clarke DH, Casals J (1958): Techniques for hemagglutination and hemagglutination inhibition with arthropod-borne viruses. American Journal of Tropical Medicine and Hygiene 7:561–573.

Clement J, Leirs H, Armour V, Ward D, Groen J, Osterhaus A, Kunz C (1992): Serologic evidence for tick-borne encephalitis (TBE) in North-American military stationed in Germany. Acta Leidensia 60:15–17.

Clement J, Leirs H, McKenna P, Armour V, Ward D, Groen J, Osterhaus A, Kunz C (1994): Parallelism between Lyme and tick-borne encephalitis (TBE) sero-epidemiology following occupational exposure in S. Germany. In Axford JS, Rees DHE (eds): "Lyme Borrehosis." New York: Plenum Press, pp 89–95.

Davenport FM, Hennessy AV (1956): A serological recapitulation of past experiences with influenza A antibody response to monovalent vaccine. Journal of Experimental Medicine 104:85–94.

De Madrid AT, Porterfield JS (1974): The flaviviruses (group B arboviruses): A cross-neutralization study. Journal of General Virology 23:91–96.

Francis T Jr (1953): Influenza, the newe acquayantance. Annals of Internal Medicine 39:203–221.

Halstead SB, Rojanasuphot S, Sangkawibha N (1983): Original antigenic sin in dengue. American Journal of Tropical Medicine and Hygiene 32:154–156.

Heinz FX, Kunz C (1981): Homogeneity of the structural glycoprotein from European isolates of tick-borne encephalitis virus. Comparison with other flaviviruses. Journal of General Virology 57:263–274.

Heinz FX, Tuma W, Guirakhoo F, Kunz C (1986): A model study of the use of monoclonal antibodies in capture enzyme immunoassays for antigen quantification exploiting the epitope map of tick-borne encephalitis virus. Journal of Biological Standardization 14:133–141.

Heinz FX, Mandl CW, Guirakhoo F, Holzmann H, Tuma W, Kunz C (1990): The envelope protein E of tick-borne encephalitis virus and other flaviviruses: Structure, functions and evolutionary relationships. Archives of Virology, Supplementum 1:125–135.

Hofmann H, Heinz FX, Dippe H (1983): ELISA for IgM and IgG antibodies against tick-borne encephalitis virus: Quantification and standardization of results. Zentralblatt für Bakteriologie und Hygiene 255:448–455.

Inouye K, Matsuno S, Yashito T (1984): "Original antigenic sin" phenomenon in experimental flavivirus infection of guinea pigs: Studies by enzyme-linked immunosorbent assay. Microbiology and Immunology 28:569–574.

Kunz C (1992): Tick-borne encephalitis in Europe. Acta Leidensia 60:1–14.

Mandl CW, Heinz FX, Kunz C (1988): Sequence of the structural proteins of tick-borne encephalitis virus (Western subtype) and comparative analysis with other flaviviruses. Virology 166:197–205.

Monath TP (1990): Flaviviruses. In Fields BN, Knipe DM (eds): "Virology," 2nd ed. New York: Raven Press, pp 763–814.

Monath TP (1994): Dengue: the risk to developed and developing countries. Proceedings of the National Academy of Sciences USA 91: 2395–2400.

Ostrow DH, Edwards B, Kimes D, Macioszek J, Irace H, Nelson L, Bartko K, Neva J, Krenc C, Mimms L (1991): Quantitation of hepatitis B surface antibody by an automated microparticle enzyme immunoassay. Journal of Virological Methods 32:265–276.

Rey FA, Heinz FX, Mandl CW, Kunz C, Harrison SC (1995): The envelope glycoprotein from tick-borne encephalitis virus at 2A resolution. Nature 375:291–298.

Stephenson JR (1988): Flavivirus vaccines. Vaccine 6:471–480.

Van Damme P, Thoelen S, Cramm M, De Groote K, Safary A, Meheus A (1994): Inactivated hepatitis A vaccine: reactogenicity, immunogenicity, and long-term antibody persistence. Journal of Medical Virology 44:446–451.